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Award Number: W81XWH-04-1-0682

TITLE: Platelet Activating Factor: A Growth Factor for Breast Cancer

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Winston-Salem NC 27157-0001

REPORT DATE: September 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-09-2006	Annual	15 Aug 04 – 14 Aug 06

4. TITLE AND SUBTITLE
Platelet Activating Factor: A Growth Factor for Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0682

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Wake Forest University Health Sciences
Winston-Salem NC 27157-0001

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Women with a diet rich in fish oils are less likely to develop breast cancer. Recent studies have shown that the ratio of two families of essential fatty acids is important in regulating many cellular processes. Nutrition is extremely important in determining this ratio since both the separate families are obtained only from the diet. The omega 3 family is enriched in fish and grains and the omega 6 family is enriched in the meats and oils typical in modern diets. The strategy of this proposal was to study the molecular mechanisms that control this effect. We focused on the synthesis of platelet activating factor (PAF). We have shown that PAF can increase the growth of breast cancer in cell cultures. Our hypothesis is that the ratio of omega 3 and omega 6 fatty acids influences the synthesis of PAF. This idea came from the fact that these fatty acids must be removed from the precursor of PAF for PAF synthesis to occur. The goal of this proposal was to test this idea and provide the knowledge that would stimulate further clinical trials using fatty acid supplementation to prevent recurrent disease.

15. SUBJECT TERMS
None provided.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	14	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction:

Breast cancer risk is decreased by a diet rich in fish oils (1). However, the molecular basis for this effect is unknown. It has been proposed that the effect is due to alterations in prostaglandins. However, we hypothesized that the n-3 fatty acids alter the synthesis of platelet activating factor (PAF) and thus alter growth and metastatic potential of the breast tumors (2, 3). The n-3 polyunsaturated fatty acids are predominantly incorporated into the *sn*-2 position of phospholipids replacing the n-6 fatty acids which include arachidonic acid (4). PAF synthesis, initiated by $cPLA_2$, uses 1-O-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine as a substrate for synthesis of lyso-PAF the direct precursor of PAF. We hypothesized that cells enriched in n-3 fatty acids will have predominantly 1-O-alkyl-2-eicosapentanoyl-*sn*-glycero-3-phosphocholine which will be a poor substrate for $cPLA_2$, as noted in earlier studies, and thus yield less PAF.

We proposed that these changes in PAF synthesis contribute to the beneficial effects of n-3 fatty acids in breast cancer. The goal of this project was to test the hypothesis that enrichment of cells with n-3 fatty acids decreases PAF synthesis and decreases the growth and metastatic potential of breast cancer cells. To test this hypothesis we determined the effect of n-3 fatty acid supplementation on the synthesis of PAF. We tested the hypothesis that n-3 fatty acid supplementation will decrease the precursors for PAF and decrease PAF synthesis. We used MCF-7 breast cancer cells which have a low content of ether-linked lipids and are not metastatic in nude mice. We have transfected MCF-7 cells with the gene for alkyl dihydroxyacetone phosphate synthase (ADS) and found that the transfectants have increased ether lipids, increased basal PAF synthesis, and grow faster.

To determine if n-3 fatty acid-altered PAF synthesis alters the growth of breast cancer cells *in vitro* we tested the hypothesis that altered synthesis of PAF will have a direct effect on cell growth. The synthesis of PAF was modified by supplementation of the cells with n-3, or n-6 fatty acids and determining the effects on cell proliferation. To confirm that the effects seen are due to PAF, we will use PAF-receptor antagonists and attempt to mimic the effects with a methylcarbamyl analog of PAF (an analog resistant to serum PAF acetyl hydrolase). The untransfected MCF-7 cells are expected to release EPA and produce eicosanoids but no produce PAF. These MCF-7 cells will be grown in culture and supplemented *in vitro* with n-3 (EPA, α-LA) or n-6 (AA or linoleic acid) fatty acids and the distribution among phospholipid species will be determined by tandem mass spectrometry. PAF synthesis in unstimulated cells was determined by tandem mass spectrometry.

Relevance: If PAF is found to be a growth and angiogenic factor for breast cancer cells, these studies can be followed up by *in vivo* studies in nude mice to assess metastatic potential. New chemopreventive strategies using n-3 fatty acid supplementation to suppress PAF synthesis could be developed. Alternatively, PAF receptor antagonists could be developed as preventive or therapeutic agents.

Key Research Accomplishments

Phospholipid Composition of Breast Tumor Cells

We examined the ether-lipid content and molecular species distribution of four commonly used breast tumor cell lines. The cells had similar distributions of phospholipid classes. However, the ether-linked subclasses were markedly elevated in the PC of MDA 231 and MDA 435 cells, but they were almost absent in MCF-7 and T47D cells (Table 1). The enrichment of the alkyl and alk-1'-enyl subclasses in PC and PE, respectively, is typical of most cells containing the ether-linked subclasses. These findings were recently confirmed by us using tandem mass spectrometry. In studies of the neutral lipids, we observed 1-alkyl-2,3-diacyglycerol in both metastatic cell lines but none in the less aggressive cells. Thus we will study the role of ether-linked lipids in combination with the n-3 and n-6 fatty acids because their metabolism is closely linked and appears to be altered in breast cancer.

Cell line	Mol % of each subclass in PC		
	Alkyl	Alk-1-enyl	Diacyl
MCF7	1.5	0.7	97.8
T47D	0.1	1.0	99
MDA 231	21.7	3.2	75.2
MDA 435	13.0	0.5	86.4

Table 1. Analysis of the subclass composition of choline phospholipids in breast cancer cell lines by tandem mass spectrometry.

We also examined the arachidonate (AA) content of the breast tumor cell lines, all grown in the same medium (DMEM). The AA was highest in the MDA 231 cells where it accounted for 23% of the acyl chains of PE and 5% of the PC chains. Corresponding values for the MDA 435 cells were 15% in PE and 1.6% in PC. Likewise, the MCF-7 and T47D cells contained 11% AA in their PE and 1.5 % in their PC. It will be important to extend these studies to determine the *sn*-2 AA content specifically; if the AA is all in the *sn*-2 position, the % AA could be up to twice the values given. This can readily be accomplished by treating the PE and PC with venom PLA_2, which specifically hydrolyzes the *sn*-2 position.

Altering the ether-linked lipid content of MCF-7 cells by transfection of alkyl-DHAP synthase.

As shown above, many highly invasive breast cancer cell lines have an increased content of ether-linked lipids (i.e. MDA-231, MDA-435). However, some less aggressive lines like MCF-7 have low ether-linked lipids. However, it is not known if the increased ether-lipid content was causing the cells to be more aggressive. Since a single enzyme, alkyl-DHAP synthase, is unique to ether lipid synthesis we hypothesized that the ether lipid content and PAF synthesis of MCF-7 cells could be increased by expression of

alkyl-DHAP synthase. To test the hypothesis that increased ether-linked phospholipids could increase PAF synthesis, we have transfected MCF-7 cells with a plasmid encoding alkyl-DHAP synthase. The original plasmid, in the pET-15b vector, was a generous gift from Professor RJA Wanders, Academic Medical Centre, Laboratory of Genetic Metabolic Diseases, Amsterdam. Our laboratory excised the alkyl-DHAP synthase from the pET-15b vector using Xho I and Xba I. The pIRES2-EGFP vector was also cut using Nhe I and Xho I. The gene fragment from the pET-15b vector was ligated into the pIRES2-EGFP vector. Note that Xba I and Nhe I restriction digests will ligate together. DNA sizing gels were run at each stage to ensure that the correct digest was obtained and that ligation had occurred. Sequence analysis was performed by the DNA sequencing core laboratory of Wake Forest University. The kanamycin resistance plasmid was transformed into XL-1 Blue competent *E. coli* (Stratagene) and expanded using 25 mg/ml kanamycin. DNA was isolated using an alkaline lysis and purification protocol. MCF-7 cells ($2x10^5$ cells / 35mm dish) were transfected with the final vector (2μg) using LipofectAMINE Transfection Reagent (Gibco/BRL) with the pIRES2-EGFP vector from Clontech or the vector plus a gene encoding alkyl-DHAP synthase. The cells were split into larger dishes and treated with Geneticin (800 μg/ml) at 48 hours after transfection. Colonies were picked, expanded, and monitored for green fluorescence. Positive clones were frozen in liquid nitrogen and assayed for alkyl-DHAP synthase by immunoblotting using and antibody to the sequence 70-123: Ac-CQESGTIPKKRQEVMKW-amide and 471-485: Ac-CEGDREKVLQHEKQVY-amide of alkyl-DHAP synthase which was prepared for us by Biosource International, Camarillo,CA. The alkyl-DHAP construct we used offers several strategic advantages. The CMV promoter fosters a high rate of transcription for a biscistronic mRNA that is separately translated into the synthase and GFP. The synthase thus retains its *N* and *C* termini and peroxisome-localizing signal. Furthermore, the expression of fluorescence in transfected cells is a strong indicator that the inserted gene, which lies upstream to *GFP*, is transcribed. Finally, the attenuated promoter for neomycin resistance favors the expression of synthase and GFP at higher levels than antibiotic resistance. The cells transfected with alkyl-DHAP synthase had a strikingly higher content of alkyl-linked molecular species than the cells transfected with the empty vector (Fig. 1, top panel) or the parental cell line (data not shown). The alkyl species are shown by the brackets in Figure 1. Each group, within a bracket, differs by unsaturation not by chain length.

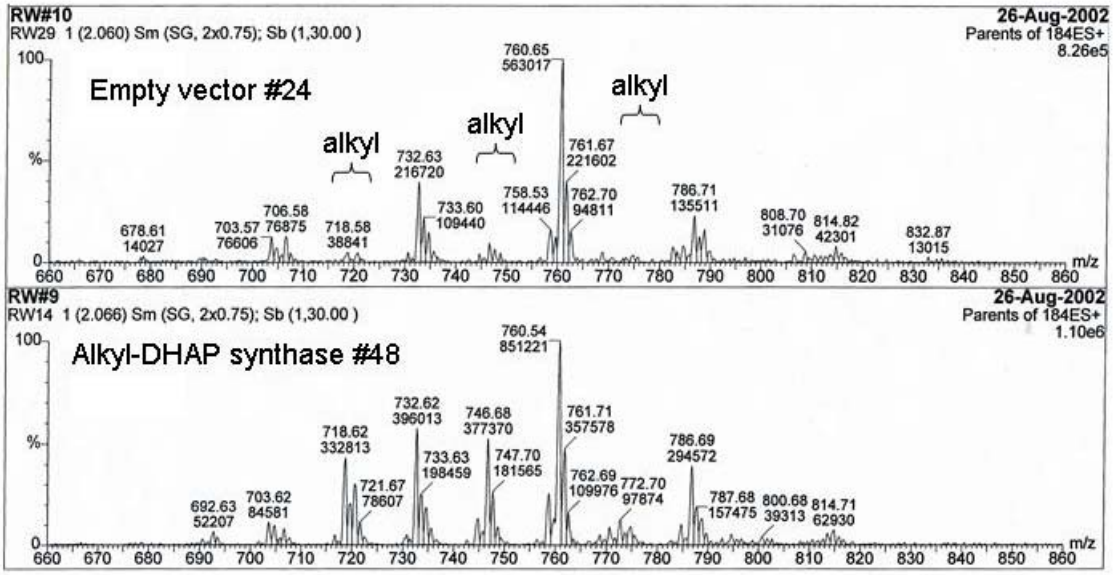

Figure 1. PC molecular species profile of MCF-7 cells. A crude lipid extract of MCF-7 cells transfected with alkyl-DHAP synthase or the empty vector were analyzed by tandem mass spectrometry as detailed in General Procedures. PC molecules were specifically detected by the unique mass/charge ratio of the protonated choline head group (m/z +184) formed upon argon-induced collision in positive ion mode. Ether-linked phospholipids differ by 14 amu less than their diacyl-linked counterparts (loss of one oxygen atom and gain of two hydrogen atoms), which makes detection and characterization of the ether-linked species easy. Each ether-linked species yields only one fatty acyl chain.

PAF synthesis is stimulated by expression of alkyl-DHAP in MCF-7 cells.

We have assayed PAF synthesis in unstimulated breast cancer cells which differ in their ether lipid content (Table 1). The cell lines with higher ether lipid levels made more PAF than the MCF-7 cells as expected (Table 2). Also the MCF-7 cells expressing alkyl-DHAP synthase produced markedly more PAF than either the MCF-7 parental cells or cells transfected with the empty vector (Table 2).

Table 2. Breast cancer cell lines were grown to 60% confluence in 100 mm dishes. MCF-7 cells used were untransfected or were stably transfected with alkyl-DHAP synthase or empty vector control. Lipids were extracted and PAF quantitated as described under General Procedures. The results presented are the average of duplicates.

Cell Line	PAF (pg/nmol total lipid)
T47D	1.00
MDA 435	0.33
MDA-MB-231	2.42
MCF-7 Parental	0.52
MCF-7 + Empty vector	0.25
MCF-7 + alkyl-DHAP synthase	4.71

PAF stimulates the growth of MCF-7 breast cancer cells *in vitro*.

The metabolism of polyunsaturated fatty acids (AA, EPA, etc) are closely linked to the metabolism of ether-linked lipids. In our preliminary studies, we have developed several MCF-7 cell lines which express increased alkyl-DHAP synthase and thus increased ether-linked lipids (see Figure 1). The transfected cell lines proliferate faster *in vitro* than the parental cells lines (or empty vector transfected

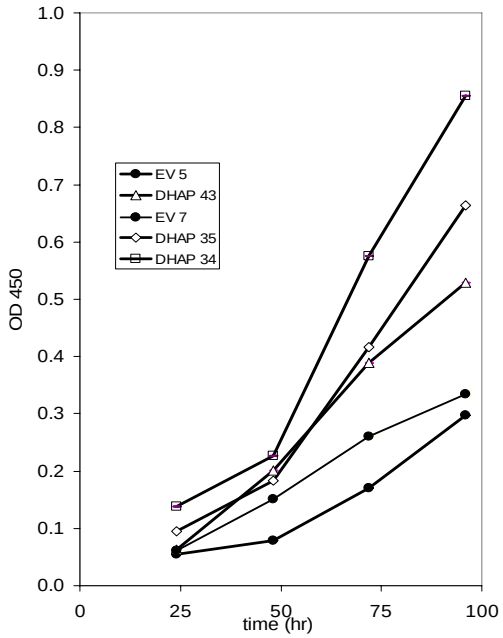

Figure 2. Expression of alkyl-dihydroxyacetone phosphate synthase stimulates growth of MCF-7 cells. When MCF-7 cells are transfected with alkyl-DHAP synthase they exhibit an increased growth rate compared to cells transfected with vector only. Three clones of alkyl-DHAP synthase expressing cells (DHAP) (open symbols) and two clones of cells transfected with the empty vector (EV) (closed symbols) are shown. The growth of the EV cells is comparable to wild type MCF-7 cells (data not shown). Cell growth was measured at the indicated times using the Cell Titer 96 assay as described by the manufacturer.

controls) (Figure 2). We hypothesized that the increased growth rate was due to synthesis of PAF. To test this idea, we used a PAF receptor antagonist which reduces proliferation of the cells which contain the transfected alkyl-DHAP synthase (Figure 3). Additionally, we have used a carbamyl analog of PAF, which is not hydrolyzed by the serum PAF acetyl-hydrolase, and found that it stimulates growth of MCF-7 cells. The alkyl-DHAP synthase transfectants were not significantly stimulated by the PAF-analog. This may indicate that the transfectants make sufficient endogenous PAF for maximum growth. Together these data indicate that PAF synthesis is important for growth of the MCF-7 cell line *in vitro*. The transfected cells also provide a tool for use in studying the role of n-3 vs. n-6 fatty acids in the control of PAF synthesis and thus cell growth.

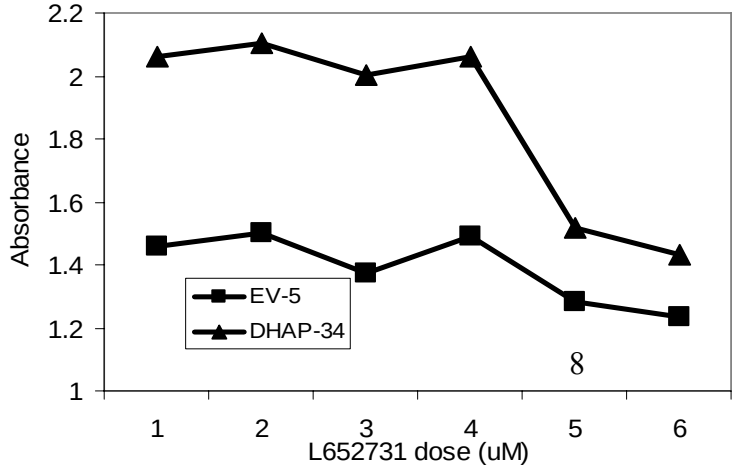

Figure 3. Inhibition of MCF-7 cell growth by a PAF receptor antagonist (L652731). MCF-7 cells transfected with alkyl-DHAP synthase (triangles) and cells transfected with the empty vector (squares) were treated with the

PAF receptor antagonist L652731 in the indicated concentrations and growth was measured after 96 hours.

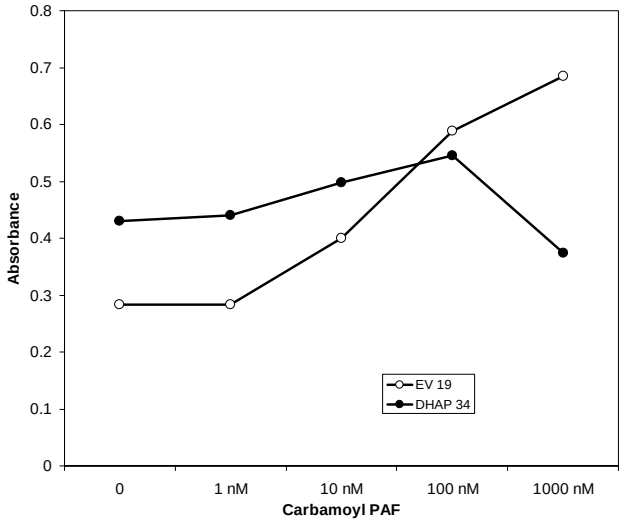

Figure 4. Stimulation of MCF-7 cell growth by carbamyl-PAF. MCF-7 cells transfected with alkyl-DHAP synthase (filled circles) or empty vector (open circles) were treated with carbamoyl PAF a non-hydrolizable analog of PAF and cell growth was measured at 48 hours. The PAF agonist stimulated the MCF-7 cells containing the empty vector but not the cells which were transfected with alkyl-DHAP synthase and had the capacity to make PAF endogenously.

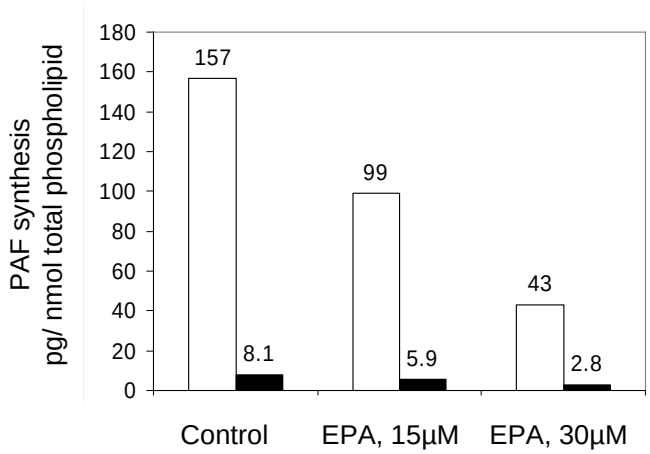

Figure 5. Supplementation of MDA-MB-231 cells with n-3 fatty acids suppresses PAF synthesis**.** MDA-MB-231 cells were grown to 60% confluence in 100 mm dishes and then incubated in serum-free medium containing no lipid supplement (*control*) or EPA (15 or 30 µM). EPA was added as a BSA complex. After 24 hr, cells were stimulated with 10 µM A23187 for 5 min.(open bars). Lipids were extracted, and PAF was quantified as described by mass spectroscopy. The data shown are the average of duplicates.

MDA-MB-231 cells which have relatively high levels of alkyl-linked lipids (Table 1) and make PAF (Table 2) were supplemented with EPA (an n-3 fatty acid) and PAF synthesis was measured in unstimulated cells (black bars) and A23187-stimulated cells (Figure 5). EPA caused a dose-dependent suppression of PAF synthesis in both the stimulated and unstimulated cells.

In summary, our data indicate that the content of ether linked lipids in breast cancer cells is important in their ability to synthesize PAF which can stimulate the growth of the breast tumor cells in vitro. Thus the ability of breast cancer cells to make PAF may be an important determinant of their ability to make autocrine growth factors.

Breast cancer risk is decreased by a diet rich in fish oils. However, the molecular basis for this effect is unknown. It has been proposed that the effect is due to alterations in prostaglandins. However, we hypothesize that the n-3 fatty acids alter the synthesis of platelet activating factor (PAF) and thus alter growth and metastatic potential of the

breast tumors. The n-3 polyunsaturated fatty acids are predominantly incorporated into the *sn*-2 position of phospholipids replacing the n-6 fatty acids which include arachidonic acid. PAF synthesis, initiated by $cPLA_2$, uses 1-O-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine as a substrate for synthesis of lyso-PAF the direct precursor of PAF. We hypothesize that cells enriched in n-3 fatty acids will have predominantly 1-O-alkyl-2-eicosapentanoyl-*sn*-glycero-3-phosphocholine which will be a poor substrate for $cPLA_2$, as noted in earlier studies, and thus yield less PAF. We propose that these changes in PAF synthesis contribute to the beneficial effects of n-3 fatty acids in breast cancer. The goal of this project is to test the hypothesis that enrichment of cells with n-3 fatty acids decreases PAF synthesis and decreases the growth and metastatic potential of breast cancer cells.

Reportable outcomes

Abstract P61-3 Era of Hope, Department of Defense, Breast Cancer Research Program Meeting, June 8-11, 2005

Alterations in platelet activating factor synthesis by n-3 fatty acids

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LeAnn Rogers#, Joseph T. O'Flaherty# and Robert L. Wykle*
Departments of Biochemistry* and Medicine#
Wake Forest University School of Medicine, Winston-Salem, NC

Breast cancer incidence is reduced in women who consume increased amounts of n-3 fatty acids (versus n-6 fatty acids) and this observation has led to the suggestion that these fatty acids are useful dietary supplements to prevent breast cancer and reduce its recurrence. Since both the n-3 and n-6 are essential fatty acids their relative levels in tissues are totally controlled by diet. The membranes of mammalian cells are composed of an array of phospholipid species that are now recognized to function as a diverse source of lipid mediators. These mediators function as both intercellular and intracellular signals and are key components of signaling cascades. The levels of ether-linked phospholipids vary greatly among cells; but, in a number of cells they are known to serve as an important reservoir of arachidonic acid (AA) and as a precursor of platelet activating factor (PAF), one of the most active mediators known. AA, an n-6 fatty acid, is the major precursor for prostaglandins and leukotrienes in people consuming a typical "Western diet". However, when the diet is enriched in n-3 fatty acids the synthesis of lipid mediators is markedly altered. When the diet is rich in n-3 fatty acids the levels of AA in the ether-linked lipids is reduced. We recently completed an analysis of the subclass composition of the choline-containing phosphoglycerides (PC) of four human breast tumor cell lines and found that the two cell lines that are not metastatic in nude mice, MCF-7 and T47D, contain only traces (0.1 to 1.5 mol%) of alkyl-PC. In contrast the highly metastatic cell lines, MDA 231 and MDA 435, contain 22 and 13 mol% respectively of the alkyl subclass in PC. Primary breast tumors had a higher ether-linked lipid content than the surrounding normal tissues. However, it was unknown if the increased ether lipids were a proximal cause of transformation or a result of altered metabolism secondary to transformation. To address this question, we transfected the

MCF-7 cell line with alkyl-dihydroxyacetone phosphate synthase and found that they have markedly increased ether-linked lipids, synthesize more PAF and exhibit a PAF-dependent increase in cell growth. Supplementation of these cells with n-3 fatty acids result in lipid alterations that decrease PAF precursors and thus PAF synthesis and cell growth.

Although not paid by this grant the following students received a portion of their training working on this project.

Jerry Saunders, PhD student in Molecular Medicine
Dawn Smith, MS student in Biochemistry and Molecular Biology

We submitted a grant entitled "Mechanism of Fish Oil Supplements in Prevention of Cancer (1P01CA106742)" using data generated during this project. The project proposed studying the effect of n-3 and n-6 fatty acids on breast cancer and prostate cancer in a mouse model. The model was PTEN -/- and developed breast and prostate cancer spontaneously. Unfortunately for the proposed studies, the mice also developed multiple other cancers and the grant was criticized based on this point and was not funded. On resubmission, we used a mouse model that was deficient in PTEN in prostate only. This required moving away from breast cancer (hopefully temporarily), and this project is now funded through 31-JUL-2011 (1P01CA106742-01-A2).

Conclusions

1. Metastatic breast cancer cells have more ether linked lipids than non-metastatic cell lines.
2. Platelet activating factor (PAF) stimulates the growth of breast cancer cells.
3. Expression of alkyl-dihydroxyacetone phosphate synthase (ADS) increases ether lipid content, growth and PAF synthesis in MCF-7 cells.
4. Eicosapentaenoic acid (EPA) inhibits the synthesis of PAF.

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Appendices:

Poster presentation: P61-3 Era of Hope, Department of Defense, Breast Cancer Research Program Meeting, June 8-11, 2005

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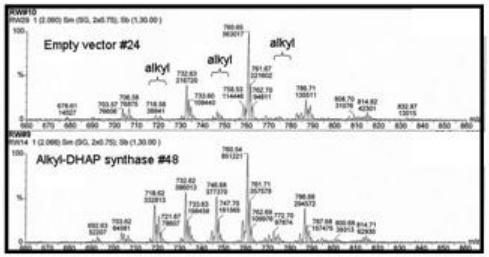
Abstract

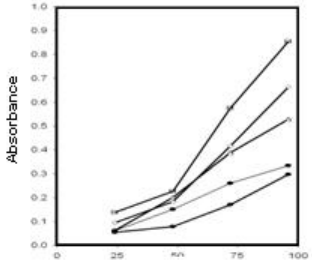
Breast cancer incidence is reduced in women who consume increased amounts of n-3 fatty acids (versus n-6 fatty acids) and this observation has led to the suggestion that these fatty acids are useful dietary supplements to prevent breast cancer and reduce its recurrence. Since both the n-3 and n-6 are essential fatty acids their relative levels in tissues are totally controlled by diet. The membranes of mammalian cells are composed of an array of phospholipid species that are now recognized to function as a diverse source of lipid mediators. These mediators function as both intercellular and intracellular signals and are key components of signaling cascades. The levels of ether-linked phospholipids vary greatly among cells; but, in a number of cells they are known to serve as an important reservoir of arachidonic acid (AA) and as a precursor of platelet activating factor (PAF), one of the most active mediators known. AA, an n-6 fatty acid, is the major precursor for prostaglandins and leukotrienes in people consuming a typical "Western diet". However, when the diet is enriched in n-3 fatty acids the synthesis of lipid mediators is markedly altered. When the diet is rich in n-3 fatty acids the levels of AA in the ether-linked lipids is reduced. We recently completed an analysis of the subclass composition of the choline-containing phosphoglycerides (PC) of four human breast tumor cell lines and found that the two cell lines that are not metastatic in nude mice, MCF-7 and T47D, contain only traces (0.1 to 1.5 mol%) of alkyl-PC. In contrast the highly metastatic cell lines, MDA 231 and MDA 435, contain 22 and 13 mol% respectively of the alkyl subclass in PC. Primary breast tumors had a higher ether-linked lipid content than the surrounding normal tissues. However, it was unknown if the increase ether lipids were a proximal cause of transformation or a result of altered metabolism secondary to transformation. To address this question, we transfected the MCF-7 cell line with alkyl-dihydroxyacetone phosphate synthase and found that they have markedly increased ether-linked lipids, synthesize more PAF and exhibit a PAF-dependent increase in cell growth. Supplementation of these cells with n-3 fatty acids result in lipid alterations that decrease PAF precursors and thus PAF synthesis and cell growth.

Choline-containing phosphoglycerides have Alkyl-, Alk-1-enyl and acyl- groups in the *sn*-1 position of glycerol. Cells vary in the relative amounts of these subclasses. We have used mass spectroscopy to show that more metastatic breast cancer cells have increased alkyl-linked species (Table 1). These alkyl species are enriched in polyunsaturated fatty acids either n-3 or n-6 depending on the cell culture media or diet (*in vitro* or *in vivo* respectively). Only the alkyl-linked subclass is a precursor for platelet activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine).

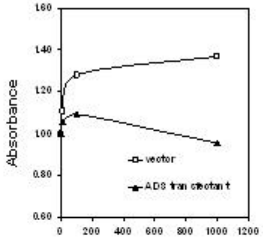
Cell line	Mol % of each subclass in PC		
	Alkyl	Alk-1-enyl	Diacyl
MCF7	1.5	0.7	97.8
T47D	0.1	1.0	99
MDA231	21.7	3.2	75.2
MDA435	13.0	0.5	86.4

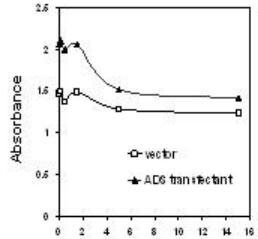
We hypothesized that the alkyl-linked lipids give a growth advantage to the more aggressive breast cancer cells. To test this idea we transfected the MCF-7 cells with a vector encoding alkyldihydroxyacetone synthase (ADS), an enzyme unique to ether lipid synthesis. The ADS transfectants have higher ether lipid content than the parental MCF-7 cells.





MCF-7 cells transfected with ADS (open symbols) grew faster in culture than the vector control cells (closed symbols). All proliferation assays used the CellTiter 96 assay from Promega.

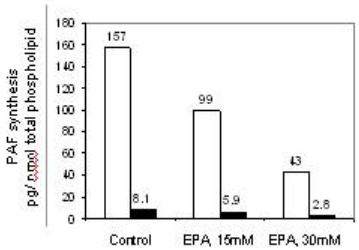




The vector-transfected cell lines were stimulated to grow by a lipase resistant PAF agonist (carbamyl-PAF).
The ADS transfectants which grew faster were inhibited by a PAF receptor antagonist.

Cell line	PAF *
MDA-MB-231	2.42
MCF-7	0.52
MCF-7 vector	0.25
MCF-7 ADS	4.71

MDA-MB-231, has higher ether lipids (Table 1) and makes more PAF than MCF-7 cells. Expression of ADS increases ether lipid content (Figure 1) and PAF synthesis.
*pg/nmol total lipid



Incubation of MDA-MB-231 cells with eicosapentaenoic acid (EPA) a n-3 fatty acid decreases PAF synthesis in response to A23187 (open bars). Constitutive synthesis was also decreased (solid bars).

Conclusions

1. **Metastatic breast cancer cells have more ether linked lipids than non-metastatic cell lines.**
2. **PAF stimulates the growth of breast cancer cells.**
3. **Expression of ADS increases ether lipid content, growth and PAF synthesis in MCF-7 cells.**
4. **EPA inhibits the synthesis of PAF.**

Research funded by: Department of Defense Breast Cancer Research Program, BC033624